APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2657–2665
0099-2240/93/082657-09$02.00/0

Vol. 59, No. 8

Interference of Humic Acids and DNA Extracted Directly from Soil in Detection and Transformation of Recombinant DNA from Bacteria and a Yeast

CHRISTOPH C. TEBBE* AND WILFRIED VAHJEN

Institut für Bodenbiologie, Bundesforschungsanstalt für Landwirtschaft, Bundesallee 50, 38116 Braunschweig, Germany

Received 11 February 1993/Accepted 3 June 1993

A two-step protocol for the extraction and purification of total DNA from soil samples was developed. Crude DNA extracts (100 μl from 5 g of soil) were contaminated with humic acids at concentrations of 0.7 to 3.3 μg/μl, depending on the type of soil extracted. The coextracted humic acid fraction of a clay silt was similar to a commercially available standard humic acid mixture, as determined by electrophoretic mobility in agarose gels, UV fluorescence, and inhibition assays with DNA-transforming enzymes. Restriction endonucleases were inhibited at humic acid concentrations of 0.5 to 17.2 μg/ml for the commercial product and 0.8 to 51.7 μg/ml for the coextracted humic acids. DNase I was less susceptible (MIC of standard humic acids, 912 μg/ml), and RNase could not be inhibited at all (MIC, >7.6 mg/ml). High inhibitory susceptibilities for humic acids were observed with *Taq* polymerase. For three *Taq* polymerases from different commercial sources, MICs were 0.08 to 0.64 μg of the standard humic acids per ml and 0.24 to 0.48 μg of the coextracted humic acids per ml. The addition of T4 gene 32 protein increased the MIC for one *Taq* polymerase to 5.12 μg/ml. Humic acids decreased nonradioactive detection in DNA-DNA slot blot hybridizations at amounts of 0.1 μg and inhibited transformation of competent *Escherichia coli* HB101 with a broad-host-range plasmid, pUN1, at concentrations of 100 μg/ml. Purification of crude DNA with ion-exchange chromatography resulted in removal of 97% of the initially coextracted humic acids. Recovery rates of soil-seeded microorganisms, carrying a mammal-derived DNA sequence as a marker gene, were 72 to 79% for *Corynebacterium glutamicum* ATCC 13032 pUN1, 81 to 85% for *E. coli* DH5-α pUN1, and 86 to 92% for *Hansenula polymorpha* LR9-Apr8, compared with that for DNA extracted from the respective pure cultures. Soil-extracted DNA was pure enough to detect 10^5 *C. glutamicum* pUN1 cells per g of soil by transformation of *E. coli* HB101, 10^4 cells of *H. polymorpha* per g of soil by slot blot DNA-DNA hybridizations, and 10 cells of *H. polymorpha* per g of soil by polymerase chain reaction amplification of the mammalian marker gene.

The extraction of total DNA from soil samples enables microbial ecologists to obtain biological material without the need to isolate microorganisms by cultivation. Since only a minor proportion of soil microorganisms is culturable on standard media (32), analysis of directly extracted DNA has the potential to detect specific genes of otherwise cryptic microorganisms (24, 26) or monitor changes in the genotypic diversity of soil microbial communities (1). When the fate of a genetically engineered organism introduced into a soil environment is being studied, direct extraction of DNA, followed by a specific quantitative detection, can potentially determine the persistence of a recombinant gene under natural conditions. This persistence does not necessarily correlate with the survival rate of an introduced organism, because recombinant DNA can be transferred into indigenous microorganisms (13, 19, 27, 30, 38) or even survive in soil extracellularly for a certain period of time (20). Thus, to understand the ecological impact of environmental releases of genetically engineered microorganisms, it is desirable to determine both the persistence of the organism and the persistence of the recombinant DNA molecule.

The importance of soil DNA and genotype analyses in microbial ecology is documented by the recent development of several extraction protocols. These protocols are either based on extraction of bacterial cells from soils or sediments and subsequent lysis as initially described by Torsvik (31), Holben et al. (11), and Steffan and Atlas (28) and further developed by other authors (12, 17) or based on direct lysis of cells in the soils or sediments followed by DNA purification (5, 10, 14, 16, 18, 23, 24, 26, 35). Higher yields of DNA from soils or sediments are usually obtained with the direct lysis method, because this method also extracts nonbacterial and extracellular DNA (29). Direct extraction of total DNA, however, always results in coextraction of other soil components, mainly humic acids or other humic substances, which negatively interfere with DNA transforming and detecting processes (20, 28, 29, 33, 34). It has been reported that those substances inhibit restriction endonucleases (11, 12, 18, 35) and *Taq* polymerase, the key enzyme of the polymerase chain reaction (PCR) (36), and decrease efficiencies in DNA-DNA hybridizations (29). The methods applied to further purify the extracted DNA, however, like the use of hydroxyapatite columns or cesium chloride-ethidium bromide density centrifugations, are time-consuming and limit the number of samples which can be analyzed. Additionally, they often result in significant losses of extracted DNA and decreased recovery rates. To utilize direct extraction methods on a routine basis, e.g., for monitoring the gene persistence in field releases of genetically engineered microorganisms, a further improvement regarding the number and sizes of soil samples that can be handled, the DNA purification procedure, and the efficiency and specificity of detection is still necessary.

* Corresponding author.

The intention of this investigation was to develop a protocol which allowed the rapid and efficient extraction and analysis of total soil DNA including recombinant DNA from taxonomically different microorganisms and from different types of soil. A two-step protocol was applied: in the first step, the aim was to extract from a soil sample as much DNA as possible (crude DNA), and in the second step, DNA was purified from coextracted soil contaminants. Specific regard was given to the investigation of the impact of coextracted substances, specifically humic acids, on gene detection efficiencies. As methods of detection, DNA-DNA hybridizations with soil DNA extracts, nonamplified and amplified by PCR, and transformation for the detection of intact plasmid molecules were applied. Genetically engineered bacteria and a yeast (*Hansenula polymorpha*) were used as tracer organisms. The organisms carried a sequence 174 bp in length which was derived from a mammalian gene coding for the peptide aprotinin. In the gram-negative organism *Escherichia coli* and the gram-positive organism *Corynebacterium glutamicum* pUN1, the sequence was located on a broad-host-range plasmid, pEK0 (7), and for the yeast *H. polymorpha* LR9-Apr8, the aprotinin gene together with a leader sequence and a termination region was integrated into the chromosome with eight tandem repeats (9). Preliminary experiments indicated that the selected mammalian gene sequence was unique in the soil environment and therefore was expected to be a suitable marker for detecting recombinant genes in total DNA extracted from soil.

MATERIALS AND METHODS

Soils. Three different soils were collected from agricultural fields near Braunschweig, Germany: a silty sand; a sandy, silty loam; and a clay silt. The soils were collected from the Ap-horizon (plow layer) in early spring when water saturation was 36.0 to 49.6% of the total water-holding capacity (WHC). Soils were air dried at room temperature to 10 to 20% WHC. After sieving (2-mm mesh size), soils were stored at 4°C in the dark for up to 6 months. The soils had the following properties. Silty sand had 81.4% sand, 15.6% silt mineral, and 3.0% clay material; pH 6.7; total organic carbon, 1.15%; total bound nitrogen, 0.067%; and total WHC, 28.9%. Sandy, silty loam had 37.5% sand, 40.8% silt mineral, and 21.7% clay material; pH 5.9; total organic carbon, 1.00%; total bound nitrogen, 0.100%; and WHC, 42.2%. Clay silt had 8.0% sand, 71.2% silt mineral, and 19.0% clay material; pH 7.1; total organic carbon, 1.97%; total bound nitrogen, 0.195%; and WHC, 27.8%. Total humic acid contents of the soils (see Results) were determined according to method 94-3.2, described by Black et al. (3).

Microorganisms, plasmids, and cultivations. The bacteria *C. glutamicum* ATCC 13032 and *E. coli* DH5-α, both transformed with plasmid pUN1, were gifts from H. Sahm, Jülich, Germany. Plasmid pUN1 is a derivative of pEK0 (6.1 kb [7]) with the aprotinin gene sequence inserted blunt ended into the multicloning site. Plasmid pUN1 is maintained in *C. glutamicum* cells at a copy number of approximately 10 per cell (13a). The aprotinin gene sequence (174 bp) is derived from the amino acid sequence of aprotinin, a protease inhibitor in bovine organs (8). The gene was designed and synthesized by Bayer AG, Wuppertal, Germany. The yeast *H. polymorpha* LR9-Apr8 (*ura* mutant) was obtained from G. Gellissen, Düsseldorf, Germany. It contained the gene sequence of plasmid pFMD130Ap chromosomally integrated with eight tandem repeats (9). Plasmid pFMD130Ap (9 kb) contained the aprotinin gene sequence flanked by an α-leader gene and a terminator sequence (4, 39). Plasmid p707 (8.4 kb), obtained from H. Wehlmann, Wuppertal, Germany, and maintained in *Saccharomyces cerevisiae* WHL292 or in *E. coli* HB101, is a derivative of pJS212 (6) with the aprotinin gene including the α-leader region and a terminator region inserted into the plasmid. *E. coli* cells were grown in Luria-Bertani (LB) broth (21); *C. glutamicum* pUN1 was cultivated in a modified LB medium (tryptone, 8 g/liter [Oxoid, Unipath Ltd., Basingstoke, Hampshire, England]; Bacto Yeast Extract, 5 g/liter [Oxoid]; NaCl, 2.5 g/liter; pH 7.0). Sterile filtrated aqueous kanamycin solutions were added (final concentration, 100 mg/liter) after autoclaving. *H. polymorpha* LR9-Apr8 was cultivated on malt medium (malt extract, 30 g/liter [Merck, Darmstadt, Germany]; peptone, 3 g/liter [Oxoid]; pH 5.6). Solid media were prepared by the addition of 1.5% agar, technical grade (Oxoid).

Soil inoculation and incubation. Soils were distributed in portions of 5 g into polypropylene test tubes (50-ml Falcon tubes; Becton Dickinson, Paramus, N.J.). For sterilization, soils were autoclaved for 1 h at 121°C and humidity was readjusted to 10 to 20% of the WHC. Soil samples were preincubated at 20°C overnight. Cell suspensions were obtained from batch culture-grown early-stationary-phase cells, which were centrifuged for 10 min at 5,000 × *g* and 4°C and resuspended in 0.85% NaCl. Cell concentrations of the suspensions were determined microscopically with counting chambers and diluted in 0.85% NaCl to the desired cell concentrations. To inoculate nonsterile and sterile soils, cell suspensions (250 to 500 μl; to result in 50% saturation of the WHC) were slowly loaded onto the soil surface. Samples were incubated for 1 h at 20°C before total DNA was extracted.

DNA extraction and purification from soils and from pure cultures. Soil samples (5 g) or pure cultures (5 ml) were suspended in 10 ml of lysis solution (0.05 M NaCl, 0.01 M Na_2 EDTA, 0.05 M Tris-HCl, pH 8.0) containing lysozyme (20 mg/ml) or in an additional 1,000 U of lyticase (Boehringer, Mannheim, Germany) for the detection of *H. polymorpha*. After being vortexed for 30 s, the soil slurry was incubated in a water bath at 37°C for 30 min with shaking and additional vigorous agitation at 5-min intervals. The tubes were then placed on ice, and 1 ml of 0.05 M NaCl–0.01 M Na_2 EDTA–0.05 M Tris-HCl (pH 8.0)–10% sodium dodecyl sulfate solution was added. To prevent foaming, 400 μl of antifoam A (Sigma Chemical Co., St. Louis, Mo.) was added. The soil slurries were transferred into agate beakers (50-ml holding capacity; Retsch, Haan, Germany) containing three agate balls and ground for 10 min in a mortar mill (Type S1, Retsch) at maximum speed. Samples were then transferred into 50-ml centrifuge tubes and immediately placed on ice. Proteinase K was added (250 U per sample), and soil suspensions were incubated at 65°C for 30 min with vigorous agitation at 5-min intervals. One volume of ice-cold, Tris-HCl (pH 8.0)-saturated phenol-chloroform (1:1) was added, and the samples were vortexed at the highest-speed setting for 30 s. After inversion of the tubes for 2 min, the suspensions were centrifuged for 15 min at 28,000 × *g* at 4°C. The aqueous upper phase was transferred to a fresh tube, and the phenolic phase was extracted with 1 volume of TE (10 mM Tris, 1 mM Na_2 EDTA, pH 8.0) and centrifuged as described above. Both aqueous phases were combined and subjected to extraction with 1 volume of chloroform-isoamyl alcohol (24:1) to remove traces of phenol. Isopropanol (1 volume) and 2 g of CsCl to enhance precipitation were added to each sample and incubated for 30 min at 4°C. The samples were

then centrifuged for 40 min at 28,000 × *g* at 4°C, and the pellets were washed with 70% ethanol. The resulting pellets were dark brown. Crude DNA solutions were obtained by resuspending these pellets in 100 μl of TE. The procedure lasted 8 h for the extraction of 12 samples of soil. To eliminate humic acids and other coextracted substances, the precipitated brownish pellets were resuspended in 10 ml of TE–1 M NaCl, pH 7.0 (diluted crude DNA). Ion-exchange columns (Qiagen-Tip 500; Diagen, Düsseldorf, Germany) with a capacity for 500 μg of double-stranded DNA were equilibrated according to the manufacturer. The diluted crude DNA solution was loaded onto the columns and washed as suggested by the manufacturer, except that the volume of washing buffer was increased from 60 to 200 ml per column. DNA was eluted with 22 ml of high-salt elution buffer. Soil DNA was precipitated with isopropanol (0.7 volume) at room temperature. Centrifugation (40 min, 28,000 × *g*, 4°C) and subsequent washing with 70% ethanol yielded DNA pellets which were translucent in appearance. The pellets were suspended in 100 μl of TE (pH 8.0) and stored at 4°C or −20°C for further analysis. Purification of crude DNA was accomplished for 12 samples in 4 h.

DNA probes, DNA-DNA hybridizations, and detection of hybridized products. Three DNA probes were used. The aprotinin gene probe (174 bp) was prepared by restriction digests of pUN1 with *Bam*HI and *Hin*dIII. The fragment was isolated by preparative agarose gel electrophoresis and purified with Gene Clean glass milk (Renner, Dannstadt, Germany). DNA was then labelled by random priming with DIG-11-dUTP (DIG labelling kit; Boehringer). The α-leader aprotinin probe (390 bp), which consisted of the whole aprotinin gene and parts of the adjacent α-leader gene, was prepared by PCR with p707 as a template. The product was labelled during PCR by including DIG-11-dUTP as suggested by the manufacturer (Boehringer). A third probe used was a 1.6-kb fragment of plasmid pUN1, which contained the aprotinin gene and 3′-adjoining plasmid DNA including a part of the kanamycin resistance gene. The fragment was prepared by restriction digest of pUN1 with *Sma*I, subsequent purification, and labelling as described above for the aprotinin gene probe. DNA was transferred from agarose gels onto positively charged nylon membranes (Diagen) with a vacuum filtration manifold (VacuBlot Transfer System, VAC-100; American Bionetics, Emeryville, Calif.). Solutions were transferred onto the same membranes with a slot blot microfiltration apparatus (Minifold; Schleicher & Schuell, Dassel, Germany). The membranes were hybridized with the indicated DIG-labelled DNA probes as suggested by the manufacturer (DIG detection kit; Boehringer). Hybridized products were detected by autoradiography (Kodak X-Omat AR; Eastman Kodak, Rochester, N.Y.).

Determination of the inhibition of DNA-transforming enzymes by humic acids. Digestions of DNA and RNA were performed under standard conditions (21). RNase (0.5 μg; DNase Free; Boehringer) was incubated in a total volume of 25 μl containing the buffer recommended by the manufacturer and RNA of phage MS 2 (1.6 μg; Boehringer) at 37°C for 1 h. Restriction endonucleases and DNase I (8 to 12 U; all obtained from Boehringer) were incubated with the appropriate DNA substrate (1 μg) for 4 h at 37°C. DNA substrates were plasmids p707, pUN1, and pBR322 (Boehringer) or uncut lambda DNA (Boehringer), depending on the enzyme tested. Humic acids (serial 10-fold dilutions and in the replicate experiment serial 2-fold dilutions) were added prior to restriction enzymes. The inhibition by humic acids was tested with both a commercially available substrate (Aldrich, Steinheim, Germany) and humic acids containing crude DNA extracts of a clay silt. Inhibition assays were analyzed after agarose gel electrophoresis and ethidium bromide staining by UV illuminations (312 nm) of the gels (21). The MIC was determined as the lowest concentration of humic acids which resulted in a different restriction pattern of DNA fragments compared with a control without humic acids. Inhibition of *Taq* polymerase from three different commercial sources (Boehringer; Promega, Heidelberg, Germany; Perkin-Elmer Cetus, Langen, Germany [source of Stoffel fragment]) by humic acids was determined by standard PCR amplifications (see below), except that T4 gene 32 protein (Boehringer) was omitted if not otherwise stated and humic acids were added in serial twofold dilutions.

PCR. Primers (20-mers; ALF1 and APR7) were designed in cooperation with R. Amore (Düsseldorf, Germany). The amplicon was a 390-bp fragment, consisting of a part of the α-leader sequence and the complete aprotinin gene. The 5′-to-3′ sequence of ALF1 was CGC AGC ATC CTC CGC ATT AG, and that of APR7 was AGC ACC ACC GCA AGT ACG CA. Both primers were synthesized by U. Ney, Jülich, Germany. PCRs were performed in 0.5-μl Microfuge tubes (Biozyme, Hameln, Germany) in a total volume of 100 μl. PCR amplifications were performed with a DNA thermocycler, TPS 20 (Landgraf, Langenhagen, Germany). Reaction solutions contained a master mix of the following composition: 1× PCR buffer (supplied by the respective *Taq* polymerase distributors)–primers ALF1 and APR7 (0.5 μM each)–deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP [200 μM each])–$MgCl_2$. For amplification of soil-extracted DNA, the following optimum $MgCl_2$ concentrations were determined: 2.0 mM for *Taq* DNA polymerase from Boehringer, 3.75 mM for Ampli*Taq* DNA polymerase, Stoffel fragment (Perkin-Elmer Cetus), and 5.5 mM for *Taq* DNA polymerase obtained from Promega (Madison, Wis.). (Note that incubation buffers from Boehringer and Promega already contain 1.5 mM $MgCl_2$.) For PCR amplifications of extracted soil DNA, 10 μl, representing 0.5 g of soil, was used as a template. DNA templates (or water for negative controls) were pipetted through a previously added overlay of the master mix with mineral oil (light white; Sigma Chemical Co.). Samples were incubated for 2 min at 95°C and then kept at 80°C. At this step, *Taq* polymerases (4 U for enzymes from Boehringer and Promega; 8 U for Stoffel fragment) were added to each tube and 35 thermocycles, each consisting of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C, were performed. Final primer extension at 72°C lasted 2 min. For the detection of low numbers of genes (fewer than 10^4/g of soil), T4 gene 32 protein (2.5 μg/100 μl; Boehringer) was added to stabilize single-stranded DNA during primer annealing. PCR products were analyzed by horizontal agarose gel electrophoresis (2% agarose in TBE [21]) followed by ethidium bromide staining and UV illumination (312 nm).

Transformation experiments. Plasmid pUN1 was used as the transforming agent. The plasmid was extracted from soil-seeded cells (*C. glutamicum* pUN1) according to the extraction protocol for total DNA, and from pure cultures, it was extracted from LB broth-grown, late-log-phase cells with Qiagen-Tip 500 (Diagen) according to the procedure described by the manufacturer. Recipients for transformation experiments were *E. coli* DH5-α cells which were made competent as described elsewhere (21). Competent cells were stored at −76°C. For transformation, aliquots of competent cells (200 μl, approximately 2 × 10^9 cells) were thawed on ice. Dimethyl sulfoxide (3 μl) was added, and the suspension was mixed briefly. Plasmid pUN1 and, where

indicated, humic acids extracted from sterile soil, both in a total volume of 10 μl, were added to each cell suspension, which was then again mixed briefly. Suspensions were incubated on ice for 30 min and heat shocked for 45 s at 42°C in a water bath. Following a 1-min incubation on ice, the cells were diluted with 1.8 ml of LB broth and incubated for 2 h at 37°C and 150 rpm on a shaking incubator. Appropriate dilutions were then plated on LB agar containing kanamycin (100 μg/ml). Plates were incubated at 37°C overnight. Numbers of CFU were determined, a random selection of colonies was analyzed for plasmid occurrence by the alkaline extraction procedure (2), and pUN1 plasmid identity was confirmed by *Xho*I digestions.

Image analysis. Images of agarose gels and autoradiographies were recorded with a charge-coupled device camera and digitally analyzed (Cybertech, Berlin, Germany).

RESULTS

Extraction of total DNA from soil. The extraction of DNA from three different soils resulted in coextraction of other soil components, which caused a brownish color in the obtained crude DNA solutions. During agarose gel electrophoresis, the colored component migrated at the same speed as a standard humic acid mixture, which indicated that the brownish color resulted from humic acids. This was confirmed by the observation that both substances exhibited the same intensity of fluorescence when gels were exposed to UV light (312 nm). Thus, comparison of UV fluorescence between coextracted humic acids and standard humic acids allowed semiquantitative determination of the concentration of humic acids in the crude DNA extracts (data not shown). The threshold amount for detectable UV fluorescence was approximately 1 μg.

The extraction procedure resulted in fragmentation of larger DNA molecules to a maximum size of 22 to 25 kb, as judged by comparison with size standards after agarose gel electrophoresis (Fig. 1). This was found for both pure culture- and soil-extracted DNA. The use of a mortar mill, which increased yields of DNA from unseeded soils by 50 to 100% (data not shown), also increased the proportion of DNA molecules with sizes below 20 kb (Fig. 1, lanes 3, 5, 8, and 10). The smaller DNA fragments originated from indigenous microorganisms, since DNA from sterile soil seeded with *H. polymorpha* LR9-Apr8 (lane 4) or other bacteria (data not shown) had sizes of between 10 and 20 kb. Purification of crude DNA (lanes 2 to 5) did not result in high losses of DNA but removed humic acids to a level below the detection limit on agarose gels (lanes 7 to 10).

Humic acids which were coextracted with the DNA represented 0.2 to 1% of the total humic acid contents of the three soils investigated. Higher proportions of humic acids were extracted from silty sand and sandy, silty loam than from clay silt (Table 1). Contaminations of the crude DNA preparations with humic acids were 0.7 to 3.3 μg/μl. Purification of the crude DNA with ion-exchange columns and ethanol washes resulted in complete decoloring of the DNA extracts. The UV absorbance ratio $A_{260/280}$ increased from 1.2 for the crude DNA to 1.8 (±0.2) for the purified DNA. The loss of DNA during the purification procedure ranged from 10 to 20%, as determined by densitometrical comparison of crude and purified DNA on agarose gels (Fig. 1). The yields of DNA from nonamended soils were in the same range for all three soils. The amount of purified DNA which could be recovered from seeded sterile soil for three different microorganisms compared with the amount of purified DNA extracted from pure cultures ranged from 72 to 92% with a maximum standard deviation of 4.3%. Recovery rates depended on the microorganism and the type of soil. For each strain, recovery rates from silty sand and clay silt were similar, but those from sandy, silty loam were lower (Table 1).

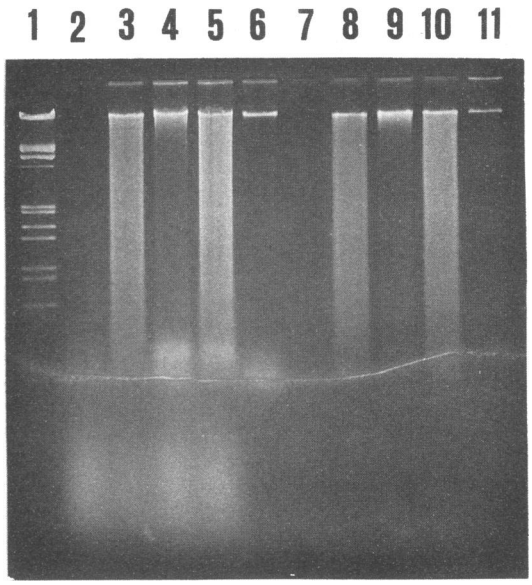

FIG. 1. Gel electrophoresis (1.0% agarose) of total DNA extracted from soil. Shown are *Eco*RI-*Hin*dIII digested lambda DNA, (size markers) (lane 1) and crude DNA extracts (lanes 2 to 5) and purified DNA extracts (lanes 7 to 10) from a clay silt. Seeded soil contained *H. polymorpha* LR9-Apr8 cells (10^6/g of soil). DNA extracted from the same number of cells from pure cultures is shown in lane 6 (crude) and in lane 11 (purified). Lanes 2 and 7, sterile and unseeded; lanes 3 and 8, nonsterile and unseeded; lanes 4 and 9, sterile and seeded; lanes 5 and 10, nonsterile and seeded. Soil-extracted DNA loaded onto the gel represented the total amount derived from 0.4 g of soil.

Effect of humic acids on the activity of nucleic acid-transforming enzymes. Further processing of extracted DNA with restriction endonucleases was possible only with purified extracts, not with crude DNA. Thus, coextracted components of the soil had an inhibitory effect on the action of such enzymes. The MICs of crude soil DNA preparations and those of standard humic acids for nucleic acid-transforming enzymes were compared on the basis of the humic acid concentrations present in the DNA-RNA digestion assays. RNase and DNase I were the most resistant enzymes identified. They could not be inhibited by crude DNA preparations in which the concentrations of coextracted humic acids did not exceed 500 μg/ml, which was well below the MIC measured with standard humic acids (Table 2). Restriction endonucleases were more susceptible to humic acids than were the previous enzymes. The inhibitory action of humic acids for different restriction enzymes was similar for crude DNA preparations from a clay silt and for standard humic acids. However, for the restriction enzymes *Sma*I, *Bam*HI, and *Sac*I, coextracted humic acids were significantly less inhibitory than standard humic acids. Differences were probably due to the fact that coextracted and standard humic acids originated from different soils and thus had different compositions. High inhibitory susceptibilities were observed for *Taq* polymerases. Depending on the commer-

TABLE 1. Total DNA and humic acid contents, DNA coextracted humic acids, and recovery of selected strains by direct extraction of three different types of soil

Soil type	Total humic acid content (mg/g of soil)	% of total humic acids coextracted in crude DNA extracts	Concn of humic acids in crude DNA extracts (μg/g of dry soil)	Yield of purified DNA from nonamended soil (μg/g of dry soil)	DNA recovery of seeded microorganisms from sterile soil in purified DNA extracts (% compared with pure culture)		
					E. coli	*C. glutamicum*	*H. polymorpha*
Silty sand	7.61	0.99	3,255	23.7	86.4	78.6	92.4
Sandy, silty loam	5.00	0.87	1,814	11.7	80.7	72.4	86.1
Clay silt	7.63	0.21	694	17.5	85.2	79.2	91.8

cial source, different MICs were obtained. In the presence of T4 gene 32 protein, however, the enzyme was eightfold less susceptible to standard humic acids. In another experiment, the influence of DNA on the inhibitory action of humic acids on *Taq* polymerase was determined. For this purpose, two different ratios of specific and nonspecific DNA were adjusted before amplification. One set of amplifications was conducted with plasmid p707 (4.8 fg corresponding to 544 copies) as the sole DNA in the reaction solution, and one set contained additionally 50 ng of nonspecific DNA of phage lambda. The MICs of humic acid for both sets of amplifications were identical, and thus, it could be concluded that there was no significant inactivation of humic acids by interactions with DNA.

Comparison of the inhibitory threshold dilutions of crude and purified soil-extracted DNA with restriction enzyme assays allowed quantitative determination of the efficiency of humic acid removal. Plasmid p707 *Xba*I digestion was inhibited in the presence of 1 μl of a 10^{-3} dilution of crude DNA. Purified DNA inhibited the same digestion only when 3 μl of a 10^{-2} dilution or lower dilutions were added (Fig. 2). Thus, 97% of humic acids were removed by purifying the crude DNA with our protocol. Inhibition assays with other restriction enzymes (*Alu*I, *Sma*I, and *Pst*I) confirmed this factor of purification (data not shown).

Interference of humic acids with DNA-DNA hybridization. To determine the effect of DNA coextracted substances on DNA-DNA hybridization efficiencies, DNA was extracted from a clay silt, which had been seeded with different concentrations of *H. polymorpha* LR9-Apr8. The DNA extracts, crude and purified, and pure culture-extracted DNA of the same strain as a control were transferred onto membranes by slot blot microfiltration. After hybridization with the aprotinin gene probe, signal intensities of pure culture-extracted DNA and purified soil DNA were similar. The detection threshold was 10^4 cells per g of soil or 4×10^3 cells (3.2×10^4 target genes) per slot (Fig. 3b and c). No hybridization signals were detectable with DNA from unseeded soils or soils seeded with fewer than 10^4 cells per g.

TABLE 2. MICs of a standard humic acid mixture and DNA coextracted humic acids from a clay silt for nucleic acid-transforming enzymes

Enzyme	MIC of the following humic acids (μg/ml):	
	Standard	Coextracted from a clay silt[a]
RNase	7,600	>547
DNase I	912	>547
*Alu*I	17.2	25.9
*Bgl*II	8.62	25.9
*Hin*dIII	8.62	25.9
*Sma*I	8.62	51.7
*Bam*HI	4.31	51.7
*Hae*III	4.31	1.62
*Pst*I	4.31	6.47
*Sac*I	4.31	25.9
*Eco*RI	2.16	3.23
*Sal*I	2.16	6.47
*Xho*I	2.16	3.23
*Sca*I	1.08	6.47
*Ssp*I	1.08	1.62
*Xba*I	0.54	0.81
Taq DNA polymerases[b]		
Source 1	0.64	0.48
Source 2	0.16	ND[c]
Source 3	0.08	0.24
Source 1 plus T4 gp 32[d]	5.12	ND

[a] In contrast to standard humic acids, coextracted humic acids from nonsterile soil contained DNA at a concentration of 1.2 μg/μg of humic acids.
[b] *Taq* DNA polymerases were obtained from different suppliers: source 1, Boehringer; source 2, Promega; and source 3, Perkin-Elmer Cetus (see also Materials and Methods).
[c] ND, not determined.
[d] T4 gp 32, T4 gene 32 protein.

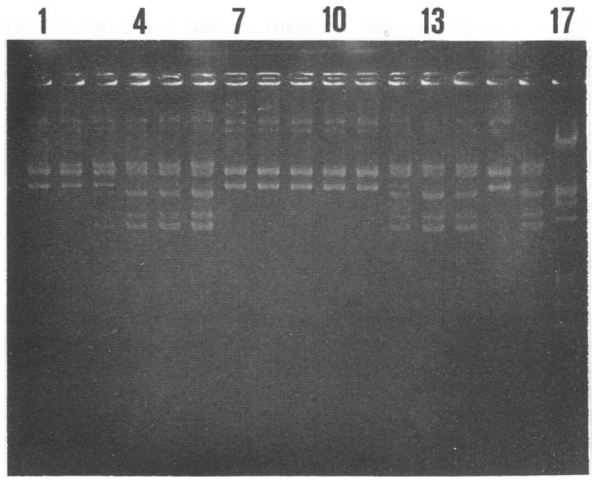

FIG. 2. Gel electrophoresis (1.0% agarose) of plasmid p707 degradation products after *Xba*I digestion for the determination of the humic acid concentrations of purified soil-extracted DNA. Shown is purified (lanes 1 to 6) and crude (lanes 7 to 14) DNA extracted from a nonsterile clay silt. The total reaction volume (23.2 μl) contained 3 μl of a 10^{-1} dilution (lanes 1 and 7), 1 μl of a 10^{-1} dilution (lanes 2 and 8), 3 μl of a 10^{-2} dilution (lanes 3 and 9), 1 μl of a 10^{-2} dilution (lanes 4 and 10), 3 μl of a 10^{-3} dilution (lanes 5 and 11), 1 μl of a 10^{-3} dilution (lanes 6 and 12), 3 μl of a 10^{-4} dilution (lane 13), and 1 μl of a 10^{-4} dilution (lane 14). Also shown are plasmid p707, undigested (lane 15); plasmid digested with *Xba*I without soil extracts (lane 16); and lambda DNA size markers (lane 17).

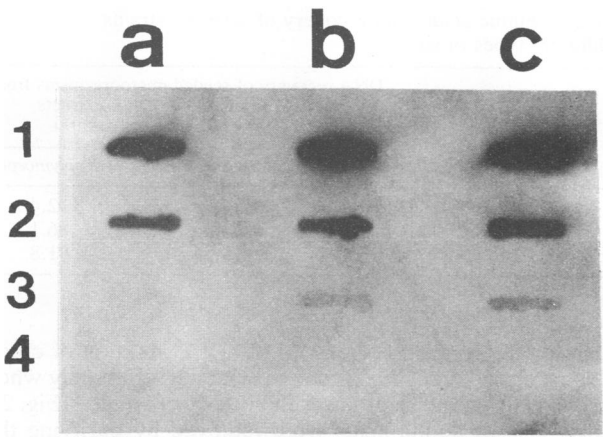

FIG. 3. Detection of *H. polymorpha* LR9-Apr8 by DNA-DNA hybridization with crude soil (clay silt)-extracted DNA (lane a), purified soil-extracted DNA (lane b), and pure culture DNA (lane c) with the aprotinin gene probe. For soil-inoculated cells, densities were 10^7 cells per g of soil (1), 10^5 cells per g (2), 10^4 cells per g (3), and 10^3 cells per g (4). Each slot represented DNA from 0.4 g of soil or the equivalent number of cells from pure culture.

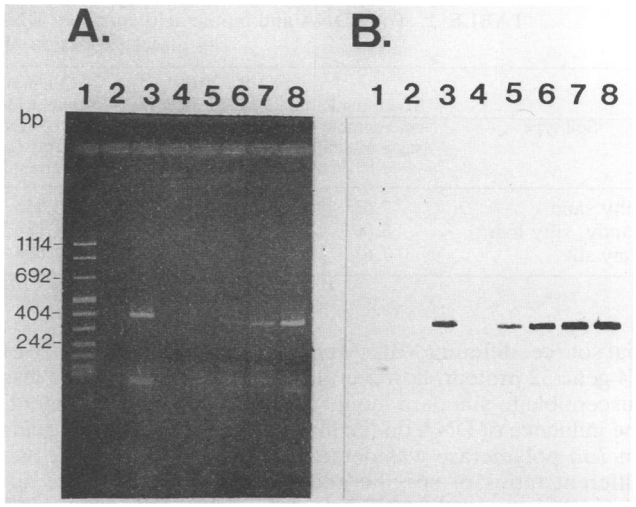

FIG. 4. Gel electrophoresis (2.0% agarose) (A) and Southern blot (B) of the PCR-amplified α-leader aprotinin gene sequence from total DNA extracted from a clay silt. Lanes 1, size standards VIII (Boehringer); lanes 2, no template DNA (negative control); lanes 3, template DNA p707 (amount at time zero, 5 fg; positive control); lanes 4, unseeded, nonsterile soil; lanes 5 to 8, *H. polymorpha* LR9-Apr8-seeded, nonsterile soil; cell concentrations per gram of soil were 10 (lanes 5), 10^2 (lanes 6), 10^3 (lanes 7), and 10^4 (lanes 8). Each lane, except standards, represented 20 μl of 100 μl of total PCR volume.

Thus, DNA from indigenous microorganisms did not show homology with the aprotinin gene probe. The presence of soil coextracted substances, which included 7 μg of humic acids for each slot, decreased the threshold of detection to 10^5 cells per g of soil (Fig. 3a). A lower detection efficiency was also observed when different concentrations of purified pUN1 plasmid DNA were hybridized with a plasmid-specific gene probe in the presence of 0.1 μg of standard humic acids transferred onto membranes by microfiltration. The decrease of detection efficiency was significant, but less than 10-fold. The signal intensities obtained when 0.1 ng of plasmid was hybridized without humic acids were higher than those obtained in the presence of humic acids (data not shown).

Detection of the aprotinin gene sequence in purified soil DNA by PCR. Amplification of nondiluted, soil-extracted crude DNA containing target DNA of *H. polymorpha* LR9-Apr8 by PCR was not possible. Purified soil-extracted DNA, however, could be amplified. Using a standard PCR protocol, it was possible to amplify the α-leader aprotinin gene sequence when 10^3 cells per g of soil, but not fewer, were present (data not shown). Two major alterations of this PCR protocol increased the sensitivity of detection dramatically. Selection of the most humic acid-resistant *Taq* polymerase and the addition of T4 gene 32 protein (Table 2) increased the threshold of detection to 10 cells per g of soil (Fig. 4). In the experiment whose results are shown in Fig. 4, DNA extracted from the clay silt was used as a template. Each PCR tube contained the total DNA of 0.5 g of soil, corresponding to approximately 8 μg of soil DNA. Even with this high background of nonspecific DNA, which was visible on agarose gels even after PCR amplifications (see upper bands in Fig. 4A, lanes 4 to 8), it was possible to amplify the α-leader aprotinin gene sequence, when 10 cells, corresponding to 80 copies or 33.6 ag of this sequence, were present, as shown by the occurrence of the 390-bp PCR product. The identity of the amplified product was confirmed by Southern hybridizations with the α-leader aprotinin gene probe (Fig. 4B). Unseeded, nonsterile soil did not result in amplification of a PCR product (Fig. 4, lanes 4). Thus, the specificity of the selected primers and the usefulness of the aprotinin gene as a marker gene were demonstrated.

Effect of humic acids on the detection of intact plasmid molecules in soil-extracted DNA by transformation. Transformation efficiencies of *E. coli* HB101 with pure culture-extracted plasmid pUN1 decreased with increasing concentrations of humic acids. Even at low concentrations (4 to 200 ng/ml), transformation efficiencies in the presence of humic acids which had been extracted from a sterile clay silt were more than 10-fold below those measured in the control without humic acids. Standard humic acid mixture did not inhibit in this range of humic acid concentrations. A relative decrease in efficiencies at 200 ng of humic acids per ml and a complete inhibition above 400 μg/ml were observed for both the clay silt-extracted humic acids and the standard humic acid mixture (Fig. 5). By using purified DNA extracted from nonsterile, *C. glutamicum* pUN1-seeded soils, it was possible to detect intact plasmid molecules by transformation when 10^5 cells per g of soil or more were present (Table 3). In contrast to the previous experiment, in which humic acids extracted from autoclaved soil were added to a transformation suspension and a slight decrease of transformation efficiencies was observed, transformation efficiencies with DNA extracted from seeded nonsterile soil were similar to those measured for controls without humic acids (see legend to Fig. 5).

DISCUSSION

To develop an efficient protocol for the extraction of DNA from soil, we characterized the effect of coextracted substances, determined the concentration of the coextracted humic acid fraction, and compared it with the effect induced by a standard humic acid mixture. All processes investigated, (i) DNA-DNA hybridization, (ii) digestions of DNA

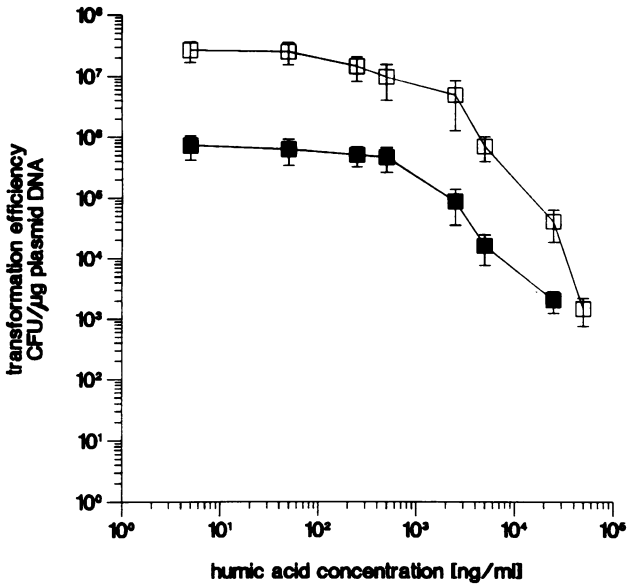

FIG. 5. Effect of humic acids on transformation efficiencies of *E. coli* HB101 with plasmid pUN1. Shown is inhibition by a standard humic acid mixture (□) and by humic acids extracted from a sterile clay silt (■). The amount of plasmid in each transformation suspension (200-µl volume) was 10 ng. Transformation efficiency without humic acids (positive control) was 2.73×10^7 CFU/µg of DNA.

by restriction endonucleases, (iii) PCR, and (iv) transformation of competent *E. coli* HB101 cells, were affected by both standard humic acids and DNA-coextracted substances.

DNA-DNA hybridizations after slot blot microfiltrations were slightly inhibited by coextracted substances and standard humic acids. An approximately 10-fold decrease in signal intensities obtained by autoradiography occurred when 7 µg of coextracted humic acids was present. Still, this allowed detection of 10^5 cells of *H. polymorpha* LR9-Apr8 per g of soil even with crude DNA. Similar thresholds of detection were found for the mycelium of *Streptomyces lividans* (40) and for *Bradyrhizobium japonicum* directly isolated from soil (11).

Restriction endonucleases were inhibited by standard humic acids in the range of 0.5 to 17 µg/ml, depending on the enzyme. Coextracted substances also inhibited these enzymes. There was a good correlation between the MICs obtained for standard humic acids and coextracted humic acid contents. Thus, inhibition of restriction endonucleases by extracted soil suspensions was probably exclusively caused by humic acids. However, in other investigations it has been reported that montmorillonite clay also can cause at least partial inhibition of such enzymes (29). The most resistant enzyme identified in this investigation was *AluI*; the most susceptible was *XbaI*. Differences in susceptibilities of various endonucleases to coextracted substances have also been described by other authors (18, 26, 35).

TABLE 3. Transformation efficiencies of *E. coli* HB101 with total DNA extracted from a clay silt seeded with *C. glutamicum* pUN1

C. glutamicum pUN1 (no. of cells/g of soil)	Total no. of transformed *E. coli* HB101 cells (CFU ± SD)	Efficiency of transformation (CFU/µg of DNA)
10^6	3,740 ± 450	5.61×10^7
10^5	840 ± 170	1.27×10^7
10^4	0	0

For PCR amplifications of soil-extracted DNA, two major inhibitory components have been identified: (i) humic acids or other noncharacterized coextracted substances and (ii) high concentrations of nontarget DNA. It has been reported that a *Taq* polymerase was inhibited by humic acid concentrations of less than 0.1 µg/ml (36). This is in accordance with our investigation, in which the most susceptible *Taq* polymerase was inhibited by 0.08 µg of humic acids per ml. However, *Taq* polymerases from other suppliers were up to eightfold more resistant. Further research may identify other heat-stable DNA polymerases which are even more resistant to humic acids and thus simplify the purification procedures necessary to obtain soil DNA for PCR amplifications. In this investigation, a recombinant gene from five cells (40 copies) of soil-seeded *H. polymorpha* LR9-Apr8 could be detected after PCR amplifications on agarose gels. In order to amplify low copy numbers of genes in directly extracted DNA, the soil extracts added as template DNA to each PCR reaction tube have to be derived from a sufficient amount of soil. In previous investigations with PCR amplifications with directly extracted DNA, template DNA in each PCR tube represented 5 mg (26, 36) or 10 mg (16) of soil. This restricted detection thresholds to approximately 10^3 cells per g of soil. With our extraction protocol, DNA was pure enough to add template DNA representing 500 mg of a clay silt, which explains the increase in the detection threshold by 1 to 2 orders of magnitude. The presence of nontarget DNA coextracted with target DNA from soil has been reported to inhibit PCR amplifications (12). In the presence of indigenous total DNA from soils, PCR amplifications were inhibited when soil DNA concentrations exceeded 1 µg/ml in the reaction mixture (5). In contrast to these reports, amplification of the *nptII* gene was possible when 0.5 µg of soil-extracted DNA was present in 50 µl of PCR solution (25). In our investigation, the aprotinin gene could be amplified successfully in the presence of 8 µg of soil DNA, when T4 gene 32 protein was added prior to PCR amplifications. It has been reported that this protein, which binds and stabilizes single-stranded DNA, has a beneficial effect on PCR amplifications (15, 22).

Transformation of competent *E. coli* cells with directly extracted DNA allows detection of biologically intact plasmid molecules. For a successful transformation, it is necessary that the transforming agent (plasmid molecule) not be damaged during the extraction procedure and that it be expressed in the recipient cell. Even though our extraction method resulted in fragmentation of larger DNA molecules and shearing as indicated by gel electrophoresis of total extracted DNA, plasmid molecules (pUN1, 6.3 kb) were still intact, so that competent *E. coli* cells could be transformed. Transformation efficiencies with soil-extracted purified DNA were as high as those with pure culture-extracted DNA. Thus, at least smaller plasmid molecules were not destroyed by the extraction procedure and could efficiently be recovered from soil-seeded cells. It has been reported that plasmid DNA which was added at high concentrations to nonsterile soil (3.6×10^{10} copies per g) was reextractable and remained active in transformation assays with competent cells of *E. coli* for a certain period of time (20). In the same study, soil components were shown to decrease transformation efficiencies. Our data suggest that this is probably a result of humic acid contamination.

The application of our extraction protocol should allow detection of other genetically engineered microorganisms, including bacteria and yeasts, by direct extraction of DNA from soil. The inhibitory effect of coextracted humic acids can be efficiently reduced in PCR-mediated detection assays by the addition of T4 gene 32 protein. Additionally, since cellular plasmid molecules were not damaged during the extraction procedure, the method has the potential to be used to monitor the presence of intact plasmids in the soil environment.

ACKNOWLEDGMENTS

We thank Katja Kortekamp for excellent technical assistance and Kornelia Smalla for reading the manuscript. The cooperative support by U. Ney, Sigrun Feldmann, and H. Sahm (Jülich); by R. Amore, G. Gellissen, and C. P. Hollenberg (Düsseldorf); and by H. Wehlman and P. Stadler (Wuppertal) is gratefully acknowledged.

This work was part of the project "Sicherheitsforschung Gentechnik," supported and funded by Bayer AG, Wuppertal, Germany.

ADDENDUM

We investigated aprotinin gene persistence in nonsterile clay silt. With our extraction protocol, intact plasmid molecules were detectable with transformation assays after 18 days of incubation, when soil was inoculated with 10^6 cells of *C. glutamicum* pUN1 per g of soil and incubated at 20°C.

REFERENCES

1. **Bej, A. K., M. Perlin, and R. M. Atlas.** 1991. Effect of introducing genetically engineered microorganisms on soil microbial community diversity. FEMS Microbiol. Ecol. **86**:169–175.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Black, C. A., D. D. Evens, J. L. White, L. E. Ensminger, and F. E. Clark.** 1965. Methods of soil analysis, part 2, chemical and microbiological properties, p. 1414–1417. American Society of Agronomy, Inc., Madison, Wis.
4. **Brake, A. J., J. P. Merryweather, D. G. Coit, U. A. Heberlein, F. R. Masiarz, G. T. Mullenbach, M. S. Urdea, P. Valenzuela, and P. J. Barr.** 1984. α-factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **81**:4642–4646.
5. **Bruce, K. D., W. D. Hiorns, J. L. Hobman, A. M. Osborn, P. Strike, and D. A. Ritchie.** 1992. Amplification of DNA from native populations of soil bacteria by using the polymerase chain reaction. Appl. Environ. Microbiol. **58**:3413–3416.
6. **Das, R. C., J. L. Shultz, and D. J. Lehman.** 1989. α-factor leader sequence-directed transport of *Escherichia coli* β-galactosidase in the secretory pathway of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **218**:240–248.
7. **Eikmanns, B. J., E. Kleinertz, W. Liebl, and H. Sahm.** 1991. A family of *Corynebacterium glutamicum/Escherichia coli* vectors for cloning, controlled gene expression, and promoter probing. Gene **102**:93–98.
8. **Fritz, H., and G. Wunderer.** 1983. Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Arzneim. Forsch./Drug Res. **33**:479–494.
9. **Gellissen, G., Z. A. Janowicz, U. Weydemann, K. Melber, A. W. M. Strasser, and C. P. Hollenberg.** 1992. High-level expression of foreign genes in *Hansenula polymorpha*. Biotechnol. Adv. **10**:179–189.
10. **Hilger, A. B., and D. D. Myrold.** 1991. Method for extraction of *Frankia* DNA from soil. Agric. Ecosyst. Environ. **34**:107–113.
11. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
12. **Jacobsen, C. S., and O. F. Rasmussen.** 1992. Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. Appl. Environ. Microbiol. **58**:2458–2462.
13. **Natarajan, M. R., and P. Oriel.** 1992. Transfer of transposon Tn*916* from *Bacillus subtilis* into a natural soil population. Appl. Environ. Microbiol. **58**:2701–2703.

13a. **Ney, U., and H. Sahm.** Personal communication.

14. **Ogram, A., G. S. Sayler, and T. Barkay.** 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol. Methods **7**:57–66.
15. **Panaccio, M., and A. Lew.** 1991. PCR based diagnosis in the presence of 8% (v/v) blood. Nucleic Acids Res. **19**:1151.
16. **Picard, C., C. Ponsonnet, E. Paget, X. Nesme, and P. Simonet.** 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. **58**:2717–2722.
17. **Pillai, S. D., K. L. Josephson, R. L. Bailey, C. P. Gerba, and I. L. Pepper.** 1991. Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. Appl. Environ. Microbiol. **57**:2283–2286.
18. **Porteous, L. A., and J. L. Armstrong.** 1991. Recovery of bulk DNA from soil by a rapid, small-scale extraction method. Curr. Microbiol. **22**:345–348.
19. **Richaume, A., E. Smit, G. Faurie, and J. D. van Elsas.** 1992. Influence of soil type on the transfer of plasmid RP4p from *Pseudomonas fluorescens* to introduced recipient and to indigenous bacteria. FEMS Microbiol. Ecol. **101**:281–292.
20. **Romanowski, G., M. G. Lorenz, G. Sayler, and W. Wackernagel.** 1992. Persistence of free plasmid DNA in soil monitored by various methods, including a transformation assay. Appl. Environ. Microbiol. **58**:3012–3019.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Schwarz, K., T. Hansen-Hagge, and C. Bartram.** 1990. Improved yields of long PCR products using gene 32 protein. Nucleic Acids Res. **18**:1079.
23. **Selenska, S., and W. Klingmüller.** 1991. Direct detection of *nif*-gene sequences of *Enterobacter agglomerans* in soil. FEMS Microbiol. Lett. **80**:243–245.
24. **Selenska, S., and W. Klingmüller.** 1991. DNA recovery and direct detection of Tn*5* sequences from soil. Lett. Appl. Microbiol. **13**:21–24.
25. **Selenska, S., and W. Klingmüller.** 1992. Direct recovery and molecular analysis of DNA and RNA from soil. Microb. Releases **1**:41–46.
26. **Smalla, K., N. Cresswell, L. C. Mendonca-Hagler, A. Wolters, and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74**:78–85.
27. **Smit, E., J. D. van Elsas, J. A. van Veen, and W. M. de Vos.** 1991. Detection of plasmid transfer from *Pseudomonas fluorescens* to indigenous bacteria in soil by using bacteriophage ΦR2f for donor counterselection. Appl. Environ. Microbiol. **57**:3482–3488.
28. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**:2185–2191.
29. **Steffan, R. J., J. Goksoyr, A. K. Bej, and R. M. Atlas.** 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. **54**:2908–2915.
30. **Stotzky, G.** 1989. Gene transfer among bacteria in soil, p. 165–222. *In* S. B. Levy and R. V. Miller (ed.), Gene transfer in the environment. McGraw-Hill Publishing Co., New York.
31. **Torsvik, V. L.** 1980. Isolation of bacterial DNA from soil. Soil Biol. Biochem. **12**:15–21.
32. **Torsvik, V., J. Goksoyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782–787.
33. **Trevors, J. T., H. Lee, and S. Cook.** 1992. Direct extraction of DNA from soil. Microb. Releases **1**:111–115.
34. **Trevors, J. T., and J. D. van Elsas.** 1989. A review of selected methods in environmental microbial genetics. Can. J. Micro-

biol. **35:**895–902.

35. **Tsai, Y.-L., and B. H. Olson.** 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol. **57:**1070–1074.
36. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
37. **Tsai, Y.-L., and B. H. Olson.** 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58:**2292–2295.
38. **van Elsas, J. D., and J. T. Trevors.** 1990. Plasmid transfer to indigenous bacteria in soil and rhizosphere: problems and perspectives, p. 188–199. *In* J. C. Fry and M. J. Day (ed.), Bacterial genetics in natural environments. Chapman and Hall, London.
39. **Waters, M. G., E. A. Evans, and G. Blobel.** 1988. Prepro α-factor has a cleavable signal sequence. J. Biol. Chem. **263:**6209–6214.
40. **Wipat, A., E. M. H. Wellington, and V. A. Saunders.** 1991. *Streptomyces* marker plasmids for monitoring survival and spread of streptomycetes in soil. Appl. Environ. Microbiol. **57:**3322–3330.